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Current status, barriers and developments in biohydrogen production by microalgae

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ABSTRACT

Biohydrogen presents tremendous potential as an alternate source of energy. Microalgae are cheaper and sustainable source of biohydrogen production. To produce hydrogen from microalgae, a two-stage method is adopted; stage-1 for carbon fixation and stage-2 for anaerobic digestion and hydrogen production. In-efficient anaerobic digestion, low hydrogen yield, and high cost are the fundamental issues of two-stage process. All these issues are attributed to the lack of understanding in key processes of hydrogen production, such as cultivation, immobilization, sulfur deprivation, and anaerobic digestion. This review gives an insight into the improvement of hydrogen yield by discussing the whole process of hydrogen production. This work also delineates the developments, barriers, and recent trends of biohydrogen production.

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1. Background

Recently, interest is growing to find cheaper and sustainable sources of energy due to escalating fuel prices and dwindling oil reserves. Sunlight is one of such sustainable and in-exhaustible sources of energy [1]. The solar radiations reach our earth containing 120,000 TW of energy, which is three times more than current human energy needs [2]. The solar energy can be captured either in solar cells or more importantly in the form of biomass energy [3]. Photosynthesis synthesizes organic compounds such as polysaccharides to produce biomass [4]. Among the available plants species, microalgae have the highest ability to perform photosynthesis, and thus, its high growth rate produces higher amount of biomass [5]. Microalgae biomass is a valuable product that produces a wide variety of biofuels such as biohydrogen, biodiesel, and bioethanol [6–8]. Among these, biohydrogen is the most appropriate choice due to its unique characteristics. It has 2.75 times more energy density than any other existing biofuels [9]. There are various methods of hydrogen production like electrolysis, pyrolysis, gasification, and steam reforming. However, biological production of hydrogen by microalgae is considered as the most favorable pathway [10]. Microalgae split water into proton (H^+) and oxygen (O_2) in the presence of light. The H^+ gets converted into hydrogen via hydrognease (a hydrogen producing enzyme). This process is called direct-photolysis [11]. The hydrogen production in this process is low because (1) H_2 and O_2 are produced concomitantly which mixes immediately, giving water as a byproduct (2) hydrognease itself is sensitive to oxygen [12,13]. This inhibitory effect can be fixed by adopting indirect bio-photolysis. Indirect bio-photolysis consists of two stages. In stage-1, the cells do photosynthesis to accumulate organic compounds (mostly glucose) and oxygen is evolved. This stage is also called aerobic stage. In stage-2, the cells degrade stored organic compounds under anaerobic condition [14]. Stage-2 is called as anaerobic stage. In two-stage process, oxygen (in stage-1) and hydrogen (in stage 2) are evolved separately. Stage-2 can be under light condition called, photo-fermentation, or without light called dark fermentation [15]. Fig. 1 illustrates the concept of two-staged hydrogen production by microalgae. Several factors affect the hydrogen yield in stage-1 and stage-2. Healthy grown cells in stage-1 produce hydrogen efficiently. Fig. 2 shows the growth of microalgae in stage-1. The growth of microalgae in stage-1 is controlled by different parameters like, light, nutrients, carbon source, temperature, pH, and bioreactor design. These parameters are equally important in stage-2 also. Immobilization and sulfur deprivation are the key intermediate steps of stage-1 and stage-2. For immobilization, the cells are suspended in a solidifying material and cut into small pieces. Immobilized cells are easy to handle, have high stability and produce more hydrogen than free cells. Sulfur deprived (S-deprived) cells yield more hydrogen than sulfur-provided cells. In the presence of sulfur, the cell synthesizes protein which suppresses the hydrogen production.

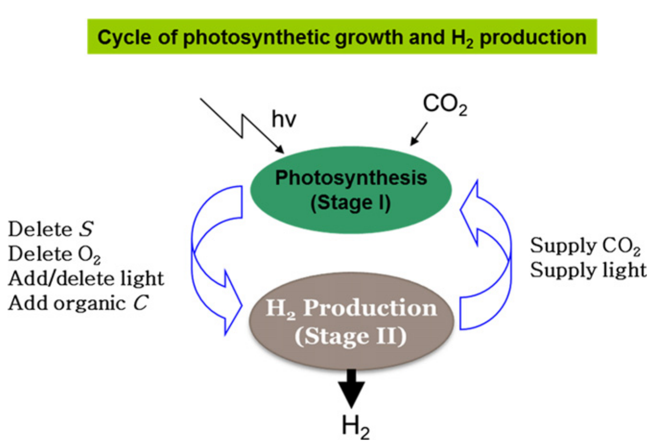


Fig. 1. Concept of two-staged hydrogen production by microalgae.



Fig. 2. Microalgae growing in photo-bioreactors.

Hydrogen is mediated by two key enzymes, *hydrogenase* and *nitrogenase*. Each enzyme activates at specific process conditions, for instance, nitrogenase is activated in the presence of light, and absence of nitrogen. Hydrogenase activates at high light intensity and pH [16]. A number of studies are available regarding hydrogen production process in anaerobic stage (stage-2), whereas, a little is focused on satag-1 [17]. An in-depth study of both stages is essential to optimize the entire process of hydrogen production and to bring further developments in this area of research.

In this context, this study presents a comprehensive report on process parameters, recent developments and future research opportunities.

2. Major enzymes in hydrogen production

In microalgae-based hydrogen production process, three enzymes are involved [18].

1. Hydrogenase.
2. Nitrogenase.
3. Uptake hydrogenase.

2.1. Hydrogenase

Hydrogenase is a major enzyme in biological hydrogen production process. Microalgae (eukaryote) and cyanobacteria (prokaryotes) contain this enzyme. In eukaryotes, hydrogenase is located in chloroplast. In prokaryotes, hydrogenase is located in cytoplasm [19]. The hydrogenase converts protons into hydrogen. In this process, protons and oxygen are produced by the splitting of water, both, in green algae and cyanobacteria. Protons are converted into hydrogen through photosystem II, photosystem I, ferredoxin, and hydrogenase. The following reaction shows the

mechanism of hydrogen production via hydrogenase [20,21]:

$$2H_2O + light \rightarrow 2H_2 + O_2 \quad (1)$$

$$H_2O - PSII - PSI - ferredoxin - hydrogenase - H_2 \quad (2)$$

The oxygen produced in this process inactivates the hydrogenase. Hydrogenase oxidizes the low redox electron carrier ferredoxin in reversible reaction. Under anaerobic conditions, the activity of this enzyme increases significantly [20].

Two types of hydrogeanses, Fe–Fe-hydrogenases and Ni–Fe-hydrogenases, are well-known in microalgae. The most efficient is Fe–Fe-hydrogenase, having 10–100 times more efficiency than Ni–Fe-hydrogenase. This hydrogenase has protein, containing Fe–Fe catalytic core. Ni–Fe-hydrogenase has selenium also in the form of selenocysteine [22]. These enzymes are sensitive to oxygen. A detailed description of hydrognease is available in Frey [22] and Mathew and Wang [19] studies.

2.2. Nitrogenase

Nitrogenase converts N_2 into ammonia and produces hydrogen. Only cyanobacteria contain this enzyme [23]. The reaction of nitrogenase enzyme is energetically inefficient due to irreversible reaction coupled to hydrolysis. Hydrogen production by nitrogenase is followed by the reaction below:

$$H_2O \rightarrow photosynthesis \rightarrow [CH_2O] \rightarrow ferredoxin \rightarrow nitrogenase \rightarrow H_2 \quad (3)$$

Cyanobacteria are divided into nitrogen fixing and non-nitrogen fixing bacteria. The mechanism of hydrogen production in nitrogen fixing and non-nitrogen fixing bacteria is followed by the following reaction [24]:

With nitrogen

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (4)$$

Without nitrogen

$$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16P_i \quad (5)$$

N_2 fixation requires ATP and reductants. The degradation of accumulated organic compounds are not ample to resume the energy requirements of nitrogenase activity. Heterocystous cyanobacteria perform oxygenic photosynthesis. Majority of them show nitrogenase activity under anaerobic or micro-aerobic conditions only [25].

Photosynthesis is a preliminary step of biological hydrogen production process. It is classified into two categories (1) oxygenic (2) non-oxygenic. The basic difference between them is the "electron donor". In oxygenic photosynthesis water is used as electron donor, while in non-oxygenic H_2S serves as electron donor [26]. Oxygenic photosynthesis is mainly performed by cyanobacteria and plants whereas, non-oxygenic photosynthesis occurs in purple sulfur bacteria. Cyanobacteria have unique characteristics to perform oxygenic photosynthesis. These are also known as blue green algae. Cyanobacteria can be found in unicellular, filamentous, and colonial form. The overall production of hydrogen in oxygenic microorganisms is the following:

$$12H_2O + 6CO_2 + light \rightarrow C_6H_{12}O_6 + 6O_2 \quad (6)$$

$$C_6H_{12}O_6 + 12H_2O + light \rightarrow 12H_2 + 6CO_2 \quad (7)$$

2.3. Uptake hydrogenase

Uptake hydrogenase consumes hydrogen [27]. It is found in all N_2-fixing unicellular and filamentous cyanobacteria. It is found in the thylakoid membranes of heterocysts [28]. The uptake hydrogenase is resistant to oxygen. Uptake hydrogenases are made oxygen tolerant through protein engineering for high hydrogen yield [27], but these enzymes require high redox potential. The uptake hydrogenase is not desired for hydrogen production [29]. The reaction of uptake of hydrogenase is the following:

$$H_2 \rightarrow uptake\ hydrogenase \rightarrow 2H^+ + 2e^- \quad (8)$$

In microalgae, three enzymes (hydrogenase, nitrogenase, and uptake hydrogenase) can be active at a time. The activities of enzymes can change by many factors; even small changes in the growth condition of microalgae can affect the activity of such enzymes.

3. Mechanism of hydrogen production

3.1. Dark fermentation

In dark fermentation, hydrogen is mainly produced by pyruvate. Pyruvate is produced by the catabolism of substrates. The substrates can be exogenic or endogenic. A wide variety of exogenic substrates can be used (the detail is given in Section 3.7). Starch in green algae and glycogen in blue–green algae are the endogenic substrates which are stored during photosynthesis. These substrates are converted into pyruvate and subsequently to hydrogen under dark condition. The pyruvate degradation is controlled by two enzymes namely, pyruvate formate lyase (PFL) and pyruvate ferridoxin oxido-reductase (PFOR). The electrons are produced by the use of substrate through TCA cycle in electron transport chain using NAD/NADH and ferredoxin. In dark fermentation, no oxygen is produced [30]. Nitrogenase is the main active enzymes in cyanobacteria in dark condition. It requires nitrogen-starved condition to produce hydrogen [31]. The hydrogen production in dark fermentation is relatively lower; only 1 to 2 mol of hydrogen are produced per mol of pyruvate. The reduced hydrogen production is attributed to the presence of uptake hydrognease, which consumes a portion of hydrogen. In microalgae, oxidative pentose phosphate pathway (PPP) produces 4 mol of hydrogen and 2 mol of acetate from 1 mol of glucose. One way to degrade acetate further into hydrogen is to re-engineer the respiratory system of microalgae. Energy supply to the microorganisms in the form of light also helps to degrade acetate into hydrogen.

3.2. Photo-fermentation

Photo-fermentation is a process in which organic compounds are degraded into small molecules in the presence of light. In photo-fermentation process, organic substrates are converted into hydrogen and carbon dioxide through ferredoxin and nitrogenase. The reaction is as follows [24]:

$$Organic\ compounds(CH_2O) + light + water \rightarrow ferredoxin \rightarrow nitrogenase \rightarrow H_2 \quad (9)$$

Das et al.[21] reported that the photosynthetic microorganisms such as *Rhodospirillum rubnum*, *Chromatium* sp, *R. capsulatus*, *R. sulidophilus*, *R. capsulata*. Miami PSB 1071, *Chlorobium limicola*, *Thiocapsa roseopersicina*, *Rhodopseudomonas sphaeroides*, *Halobacterium halobium*, *Rhodobater sphaeroides*, *Rhodopseudomonas plaustris*, can produce hydrogen via photo-fermentation. They use light as an energy source to degrade stored organic compounds (starch or glycogen) into hydrogen [30,32]. Purple non-sulfur (PNS) bacteria can produce hydrogen from organic substrates such as acetic acid, glucose, fructose, succinate, and malic acid [33]. PNS have high substrate conversion efficiencies and low sensitivity towards oxygen [27]. Majority of PNS bacteria produce hydrogen by using nitrogenase enzyme. Their unique metabolic abilities and the deficiency of PS-II reduce the inhibitory effect of oxygen on hydrogen production [34]. Phototrophic bacteria require organic or

inorganic carbon source to produce electrons during fermentation. Different types of organic wastes have been used to produce hydrogen by photo-fermentation [35]. Hydrogen production via photo-fermentation is influenced by several factors, such as light intensity, wavelength and illumination pattern [34]. Hydrogen production increases with increase in light intensity. Increase in light intensity causes photo-inhibition. As a result, low oxygen is produced and hydrogen production increases. The requirement of light to carry out photo-fermentation depends upon the type of microorganisms [21].

3.3. Immobilization

"An immobilized cell is defined as a cell that by natural or artificial means is prevented from moving independently of its neighbors to all parts of the aqueous phase of the system under study" [36]. Several techniques of immobilization have been introduced like affinity immobilization, adsorption, liquid confinement, entrapment. Kosourov et al. (2002) have described the procedure of cell immobilization using alignate. Microalgae cells are washed once by centrifugation at 3000*g* for 3 min in double-distilled water. The cells are suspended in double-distilled water and mixed thoroughly with sterile 4% alginate (sodium alginate), using the formulation ratio: 0.25–2 g wet cell weight, 0.5 mL H_2O, and 1 mL 4% alginate [37]. The cells are solidified at 4 °C and cut into pieces (a size of ~1 cm^3).

The cultivation of immobilization microalgae cell has drawn considerable attention due to its versatile applications. To cultivate immobilized cells, continuous agitation is essential to keep them suspended and to avoid the cells to form clumps. The clumps formation obstructs the cell growth due to limited gas diffusion and light permeation. Conventional agitation system causes the breakage and structural degeneration of vegetative cell that also cause cessation of bio-photolysis in culture medium [38].

Immobilized cells can be used to capture CO_2 at high rate and converting it into organic compounds. In immobilized state, the cells are divided evenly in microalgae culture medium. Studies found that, the chlorophyll content of immobilized cells remain stable even after 90 days. In contrast, free cells can be stable only for seven days. Brouersf et al. (1986) reported the hydrogen production by immobilized of *A. azollae* in polyvinyl foam. The hydrogen production in immobilized cells was higher (13 pmol H_2/per mg chlorophyll) than free living cells (8 pmol H_2/per mg chlorophyll) [39].

The age of cell immobilization also determines its stability and potential to be used in subsequent processes like biohydrogen production or metal adsorption [40]. According to Mallick, immobilized microalgae *Chlorella homosphaera* have ability to remove Cd, Zn, and Au from wastewater [36]. Garnham et al. reported to remove Co, Zn, and Mn using immobilized *Chlorella Salina* [41]. Wilkinson et al. recovered mercury upto 90% (initial concentration of 1 mg/L) which was higher than the free cell system [42].

Nevertheless, distinct advantages of immobilization, there are several issues to address for its application in hydrogen production system. Mallick et al. reported, the lag phase during cell growth was longer for immobilized cell than free cells [36]. A fraction of microalgal cells becomes inactive during immobilization process. High packing density limits the exposure of light and air, and thus, it inactivates the cells. As a result, photosynthesis (of inner cells) hinders and the cells become less active. To overcome the limitation of light, the photo-bioreactors are illuminated internally. Rashid et al. used optical fiber as light source to illuminate immobilized cells of microalgae. The optical fiber was immersed inside the bioreactor to improve light permeation. However, the use of optical fiber (> 12 h) raised the temperature (up to 45 °C) inside the bioreactor causing the cells death. The development of cool fluorescent optical fibers and their use in microalgal systems can be an intriguing approach to prove the significance of immobilized cells for hydrogen production. For the immobilization of microalgae, the size of cells-aggregate (here we termed as cube) is important to consider [43]. Rashid et al. have adopted two-staged hydrogen production using immobilized cells [44]. In stage-1, the immobilized cells were suspended in growth medium under light for photosynthesis. In stage-2, the cells were subjected to anaerobic condition for hydrogen production. Fig. 3 shows the immobilized cells in anaerobic condition. In both stages, the cube size is critical. In case of big sized cube (> 1 cm^3), a high air flow rate is required to keep these cubes in motion. Otherwise, the cells settle down at the bottom and photosynthesis hinders due to improper air and light supply. Similarly, in stage-2, a continuous stirring is required for efficient hydrogen production, which is possible by keeping a proper cube size. On the other hand, a cube with small size (< 1 cm^3) loses its viability after sometime (~24 h) due to its continuous striking with air-bubbles. Song et al. (2011) produce hydrogen in a cyclic manner (from stage-1 to stage-2 and again back to stage-1) with 1 cm^3 sized immobilized cells [44]. The immobilized cells were able to produce hydrogen up to three cycles. Further investigations on cube size and its effect on hydrogen production are still needed.

3.4. Sulfur deprivation and anaerobiosis

Sulfur deprivation exerts distinct effects on hydrogen production. Sulfur deprivation stops the synthesis of methionine, and cysteine. As a result, oxygen evolution is restrained and respiration overrides the photosynthesis to produce hydrogen. The process of sulfur deprivation is quiet simple. The cells are transferred from sulfur rich medium to S-deprived medium. Rashid et al. (2011) used S-deprived cells for hydrogen production [45]. They transferred the cell from sulfur added MA-medium to S-deprived MA-medium.

In sulfur depleted medium the anaerobic condition is achieved early [46]. Improvements in sulfur deprivation are possible by washing the cells multiple times.

The rate of hydrogen production depends on the timing of sulfur deprivation. Sulfur deprivation is adopted just before subjecting the cells in anaerobic condition. Laurinavichene et al. claimed that the duration of hydrogen production also prolongs in S-deprived condition without decreasing its rate [47]. To date, various approaches have been reported, describing the effect of light on sulfur deprivation. The rate of oxygen evolution and



Fig. 3. Immobilized cells of microalgae under anaerobic incubation.

CO_2 fixation declines significantly within 24 h in the presence of light [48]. Kosourov et al. [46]reported, after 10–15 h of anaerobiosis, S-deprived algal cell induce a reversible hydrogenase and start to evolve hydrogen gas under light. Reports have been published showing the alternate cycles of hydrogen production and photosynthesis by S-deprived cells [45]. Hydrogen production can be achieved efficiently up to three cycles if the light is provided throughout the sulfur deprivation process [43]. It is also claimed that enhanced hydrogen production can be achieved in complete dark and complete light conditions. However, both conditions give different efficiencies. Without light supply only two cycles of hydrogen can be completed. Our previous work [44], also showed, hydrogen production was maximum in complete light condition and minimum in dark. However, duration of light supply can manipulate the cost of sulfur deprivation. It was found that the light supply during first 24 h of sulfur deprivation serves the same purpose as if in complete light condition [49]. High light intensity (>300 μmol/m^2/s) causes photo-inhibition and limits photosynthesis.

Sulfur deprivation under dark has been claimed to be more efficient by some studies [15]. In dark condition, photosynthesis process stops and hydrognease activity starts due to the depletion of oxygen present in microalgae medium. During respiration, stored organic compounds generate electrons. The protons (H^+) act as electron acceptor to produce hydrogen via ferredoxin [24]. In dark condition, hydrogen is produced by fermentation. Low oxygen production in dark does not affect hydrogeanse activity greatly, and thus, high hydrogen yield is obtained. Likewise, it is predictable that even under partial light condition; the hydrogen yield would be lesser than in dark condition. Under low light condition, photosynthesis is not completely stopped and oxygen is produced to lower the activity of hydrognease [9]. On the contrary, high light condition causes photo-inhibition to elicit high hydrogen yield. However, a combination of dark and light condition can be economically viable.

Anaerobiosis in sulfur deprivation also depends upon the cultivation condition. For instance, the cells grown with high CO_2 concentration can attain early anaerobiosis as compared to low concentration. The possible explanation can be, as under high CO_2 concentration, the cells consume all the sulfur available. These cells yield produce more hydrogen due to the absence of oxygen. Microalgae collected during late-exponential phase and stationary phase, can achieve early anaerobiosis compared to those cells collected during exponential phase. Cells cannot consume the sulfur completely in exponential phase, and this un-consumed sulfur in cells lowers the hydrogen yield. Kosourov et al. proposed, the periodic addition of sulfate would restore H_2 production. Addition of sulfur re-establishes higher rates of electron transport through oxygenic electron transport chain [50]. Another study discovered, slows down the hydrogen production rate slows down for a certain time but restores its original rate after two days. The pH affects hydrogen production during sulfur deprivation, and thus, pH needs to be maintained. [51]. Some factors such as the fixation of CO_2, formation of glucose and O_2, raise initial pH, whereas the formation of organic acids reduce the pH [52] Culture density plays a typical role in establishment of anaerobic condition in sulfur deprivation [53]. Light penetration decreases in highly dense culture and it causes self-shading of the cells. Resultantly, photo-inhibition slows down, and it delays anaerobiosis. Further research is required to understand the complexity of sulfur deprivation process, and to improve hydrogen productivity. Though sulfur and nitrogen deprivation play a significant role in hydrogen production, but we could find studies related to sulfur deprivation only. The effect of nitrogen deprivation or starvation has not been illustrated yet despite the fact that nitrogen is also a protein source [54] and helps to produce oxygen. The microalgae cells accumulate more lipids than protein under nitrogen-starved conditions, and vice versa. However, hydrogen production requires accumulated carbohydrates than the proteins. Therefore, algal cells are cultivated under nitrogen starvation to prepare a feedstock for enhanced hydrogen production, protein synthesis should be suppressed which is possible by nitrogen starvation.

3.5. pH

Hydrogen production process significantly depends upon pH. A subtle change in pH can change the end products (CO_2, acetate) of anaerobic process [55]. During photosynthesis, initial pH decreases due to the formation of carbonic acid as a result of a chemical reaction between CO_2 and water. After certain time (1–3 days), pH increases due to the evolution of oxygen via photosynthesis. Generally, microalgae grow at a pH range of 5.0–9.0 [45]. High culture pH shortens the lag time of hydrogen production and increase its rate [56]. Any changes in pH, alter metabolic pathways of micro-organisms in hydrogen production process. For example, at low pH, the hydrogen is produced by hydrogenase via pyruvate–ferredoxin–oxidoreductase pathway. This route can suppress the activity of Fe-hydrogenase [57,58]. As a result, the microorganisms metabolize acetyl-CoA through energy efficient path, leading to acetate and ATP production, rather than hydrogen [56]. In one study, it was documented that mixed culture of fermentative bacteria could degrade 90.3 to 99.3% glucose at pH 4.0 to 5.5 which yielded 2.1–2.3 mol-H_2/mol-glucose [59]. In mixed microbial flora, sucrose degradation increased with pH. The maximum efficiency (95%) was found at pH 9.0 [60]. This fact can be explained in terms of enzyme activity; hydrogen producing enzymes (hydrogenase and nitrogenase) are sensitive to pH. Initially, protons, generated by the degradation of endogenic or exogenic carbon source and by the splitting of water, are converted into hydrogen. Later, proton concentration increases, a few of them are entrapped by hydrogenase or nitrogenase (depending upon the light condition applied) and get converted into hydrogen, rest of those remain un-reacted. At low pH value (<5.0), hydrogen producing enzyme inactivates, reducing hydrogen production rate. Another possibility of low hydrogen productivity associated with pH can be that during sulfur deprivation final pH reaches at different levels at different time, changing intermediate metabolic byproducts. Production of CO_2 as a final product renders high productivity, unlike the production of acetic acid or methane [56]. For instance, at elevated pH (>7.0) methane or acetic acid is produced at relatively low pH. Kosourov et al. found, during S-deprived green algae *Chlamydomonas reinhardtii* hydrogen production rate was high at 7.7 and decreased at 6.5 [17,46]. They related the pre-domination of alternative metabolic pathways with pH. It was reasoned that metabolic byproducts were due to the activation or inactivation of photosynthesis activity [61]. The pH value during photosynthesis and fermentation also depend upon the type of microalgae specie. Marine algae require different pH than fresh algae due to low nitrate requirement in former case [62]. Nitrate uptake along with carbon fixation are the main factors of pH alteration. Antal et al. work showed the dependence of hydrogen production on pH of the medium. [40,63]. They found the optimal pH for H_2 production using *Gloeocapsa alpicola* was 6.0–7.5 [63]. pH dependent optimization of hydrogen production process in photosynthetic and anaerobic phase will certainly give better insight for enhanced hydrogen yield. The production of undesirable intermediate metabolic products can be controlled by developing a correlation between photosynthetic byproducts in stage-1 and intermediate byproducts during stage-2, with pH. Extensive research is available on pH optimization in stage-1, but only few studies deal with the pH effect during stage-2. Table 1 shows the pH values for different microalgae species in anaerobic condition.

Table 1
The use of different substrates at various pH for hydrogen production.

Substrate	Optimal pH	Organism	References
Malt extract	8.0–9.0	*Chlorella vulgaris*	Rashid et al., 2011
Glucose	5.5	Mixed culture	Hallenbeck et al., 2002
Glucose	5.0–6.0	*Enterobacter cloacae*	Das et al., 2001
Glucose	6.7	*Clostridium butyricum*	Chen et al., 2008
Sucrose	6.0	Seed sludge	Lee et al.,2002
Sodium bicarbonate	5.0–5.5	*G. aplicoa*	Antal et al.,2005
Glucose	6.9	*Chlamydomonas reinhardtii*	Kosourov et al.,2002
Glucose	6.13	*Enterobacter aerogenes*	Jo JH et al., 2008

3.6. Light

Light is the most important component of biohydrogen production process [64]. Light helps in photosynthesis to reduce carbon into starch or glycogen. Light is also an integral part of photo-decomposition. In microalgae cultivation, light demand increases over time. An appropriate light supplement is necessary for healthy growth of microalgae. High light supplement in early growth stage can cause photo-inhibition or wastage of energy due to excessive light. Therefore, incremental addition of light is suggested. In incremental light supplement, the cells do not suffer photo-inhibition at early stage (lag phase) and light deprivation at later stage (exponential or stationary phase). Das et al. [27] showed, about 30% of the light cost can be saved by incremental light supply.

Manipulations of light demand in anaerobic phase can also increase the hydrogen yield. Tsygankov et al. used 25 $\mu E/m^2/s$ (as low light) of light during photosynthesis and 110–120 $\mu E/m^2/s$ (as high light) in anaerobic condition [65,66]. This pattern of light improved the hydrogen yield significantly. High light is needed at initial stage of anaerobic condition [43]. Under high light, anaerobic condition is achieved earlier than low light [66]. Laurinavichene et al. found that 30–40 $\mu mol/m^2/s$ was the optical light intensity for *C. reinhardtii* [47]. *R. sphaeroides* O.U requires a light intensity of 270 W/m^2 for maximum hydrogen production. Light conversion efficiency of *R. sphaeroides* O.U decreased from 1.11% to 0.25% as light increased from 88 to 405 W/m^2. The decrease in light conversion efficiency, however, did not change the hydrogen yield [67]. For *R. plaustris*, 680 $\mu mol/m^2/s$ is reported as the optimal light intensity [68]. Unfortunately, various units of light intensity are used in microalgal studies. Therefore, it is hard to make a direct comparison of light intensities, required for different microorganisms.

The addition of di-chlorophenyl di-methyl urea (DCMU) at high light intensity can further improve the hydrogen production. [47]. Detail study on DCMU effect is not done yet. Hydrogen yield is also affected by the wavelength of light. Uyar et al. [67]stated that the light wavelength in the range of 750–950 nm are not suitable for hydrogen production. However, a wavelength of 20–30 nm is most appropriate for microalgae cultivation and hydrogen production. Different light sources have different wavelengths. Light emitting diodes (LEDs) have shorter wavelengths (20–30 nm) than monochromatic lights. Using LEDs as light source, show high hydrogen yield than other light sources. Red and blue color of LEDs light gives more hydrogen than white. UV irradiation with monochromatic light is reported to improve the hydrogen [69].

Light influence the lag time of hydrogen production also. Khanal et al. demonstrated that the lag time was decreased under light condition and hydrogen yield was improved [56]. The hydrogen production is accompanied with the pattern of light supplement. Uyar et al. found that the hydrogen was produced during light period, stopped in dark and restored when illumination started again [34]. They used modified medium of Biebl and Pfennig as nutrients for the cells growth [70]. In hydrogen production experiments, the nitrogen source was sodium glutamate (0.002 M) and the carbon source was malate (0.0015 M). Markov et al. asserted, only 15–30 min exposure of green algae *C. reinhardtii* cells to high light intensity (2000 $\mu mol/m^2/s$) causes photo-inhibition [71]. Tsygankov et al. examined an increase in hydrogen production by 4 h light exposure in anaerobic condition. [65] Kim et al. correlated hydrogen production with residual sulfate in S-deprived medium. They found that the consumption rate of sulfur was accelerated with increasing light intensity and reach to maximum at 200 $\mu mol/m^2/s$ [72]. The investigations showed, initiation of hydrogen production depended on the consumption rate of sulfate [72].

As we stated earlier, under high light intensity (300 $\mu mol/m^2/s$) the microalgae photosynthesis stops due to photo-inhibition and no oxygen is produced. So, high light is helpful to establish anaerobic condition for high hydrogen yield [72]. Chlorophyll concentration increases with increasing light intensity. Due to high chlorophyll concentration at high light intensity, more electrons are released. The released electrons combine with protons to generate hydrogen. The pivotal role of light in algal growth and hydrogen production will remain a big debate due to its huge variations in demand at each step of photosynthesis and hydrogen evolution [34]. The effects of light on microalgae growth are well studied in the field of biodiesel production by microalgae; the optimization of light characteristics in biohydrogen related is quite limited. Conclusively, the complex nature of light in biohydrogen should be explored in photosynthetic stage (stage-1) as well as in anaerobic phase (stage-2) of biohydrogen production process.

3.7. Carbon source

Carbon source is an indispensable constituent of hydrogen production process [73]. Microalgae store carbon in the form of starch or glycogen during photosynthesis and use them in anaerobic condition. Microalgae cells can accumulate only a limited amount of glycogen and starch. Ultimately, low hydrogen is obtained. A significant increase in hydrogen yield is possible by introducing exogenic carbon source in early phase of anaerobiosis [74–76]. A wide variety of exogenic carbon sources can be used by microalgae for hydrogen production such as glucose, fructose, sucrose, malt extract, malic acid, acetate, and organic wastewater [43,77,78]. The yield of hydrogen varies with the type of carbon sources and algal strain.

The selection of carbon source is important for microalgae cultivation also. Microalgae can use inorganic carbon (CO_2) and organic carbon source (glucose, mannitol, acetate, sucrose) [68,79,80]. Microalgae grown under heterotrophic condition (using organic carbon) could have more potential to produce hydrogen than in autotrophic condition (inorganic carbon). In heterotrophic condition, high biomass is achieved. Moreover, in heterotrophic mode of cultivation organic wastes, as cheaper carbon sources can be used. Unlike autotrophic condition, light is also not required in

Table 2
Hydrogen yield by different microorganisms using various substrates.

Organism	Substrate	H_2 yield	References
Chlamydomonas reinrardtii	Acetate	1.7 mol/mol	(Gibbs et al.)
***Anabaena* sp CH_3**	Fructose	0.0016 mol/200 ppm	Chen et al.,2008
	Sucrose	0.001 mol/200 ppm	
	Glucose	0.004 mol/200 ppm	
Enterobacter ***cloacae***	Glucose	2.2 mol/mol	Das et al., 2001
Clostridium butyricum	Hexose	1.63 mol H_2/mol hexose	Wang et al., 2008
***Rhodopseudomonas* sp**	Vegetable starch	0.0013 mol/g of substrate	Kumar et al., 2000
***Rhodopseudomonas* sp**	Dairy wastewater	0.016 mol/g of substrate	Gaffron et al., 1944
***Citrobacter* sp. Y19**	Glucose	2.49 mol/mol of substrate	Oh et al., 2003
Thermoanaerobacterium ***thermosaccharolyticum* PSU-2**	Sucrose	2.53 mol/mol of substrate	Thong et al., 2008
Thermoanaerobacterium ***saccharolyticum* JW/SL-YS485**	Xylose	0.88 mol/mol of substrate	Shaw et al., 2008
Thermotoga ***maritima* DSM3109**	Glucose	1.67 mol/mol of substrate	Nguyen et al., 2008
Thermotoga ***neapolitana* DSM4359**	Glucose	1.84 mol/mol of substrate	Niel et al., 2002
Clostridium ***beijerinckii* L9**	Glucose	2.81 mol/mol of substrate	Lin et al., 2007

heterotrophic cultivation. Therefore, heterotrophic cultivation can be cheaper than autotrophic cultivation [81,82]. Bacterial contamination is a serious concern in heterotrophic growth system [83,84]. As soon as organic carbon is introduced into the medium, bacterial growth starts which outperforms the microalgae and consumes all the nutrients. Antibiotics are needed to control the contamination at this stage. In biodiesel production process by microalgae, this issue has been addressed, however, in hydrogen production it is not reported at all, to the best of author's knowledge.

In biohydrogen production process; the effect of carbon source on microalgae cultivation is not well explored yet; however, remarkable research is available regarding their effect during anaerobic condition. Wei et al. (2011) used glucose, sucrose, fructose and malt extract as substrates for *Microcystis aeruginosa* (a cyanobacteria) and *Chlorella vulgaris* (green algae). Malt extract turned the maximum hydrogen yield of 1300 mL/L of microalgae medium [45]. Chen et al. used *Anabaena* sp strain CH_3 to produce hydrogen by using glucose, fructose, galactose, and sucrose as substrate with a concentration of 200 mg/L [85]. The preferred substrate for hydrogen production in *Anabaena* sp were fructose and sucrose producing 0.0016 and 0.001 mol of hydrogen, respectively. *R. plaustris* produced 214 mL of hydrogen at 0.011 M of acetate [68]. Table 2 shows the yield of hydrogen reported by the researchers using various types of microorganisms [86].

Carbohydrate rich industrial effluent such as dairy industry, olive mill, waste baker's yeast and brewery waste can be used as carbon source. Wang et al. used molasses as nitrogen and carbon source for *Clostridium butyricum* W5 [87]. The molasses proved efficient and cheaper source of nitrogen and carbon. The highest hydrogen yield of 1.63 mol H_2/mol-hexose was obtained using 100 g/L molasses and $NH_4NO_3 \cdot 1.2$ g/L [87] Palm oil mill effluent (POME) and sludge compost were found to produce significant amounts of hydrogen by anaerobic micro flora [88]. The maximum production of yield of hydrogen per decomposed glucose was 2.1 mol/mol-glucose at 50 °C by sludge compost [89]. Hydrogen production by *Enterobacter aerogenes* was investigated in a batch system, the maximum hydrogen production rate of 425.8 mL H_2 was obtained under the optimum condition of glucose concentration (0.118 M) [90].

4. The economics

Biological hydrogen production has been the focus of research since long. Only a limited number of economic analyses of biohydrogen processes are available. Lab scale studies on various feature of hydrogen production have been carried out but the feasibility of its application at pilot scale is not recognized yet. The experimental studies have shown that dark fermentation is an economical way but it yields very low amount of hydrogen. On the contrary, photo-fermentation is more efficient method but it costs a lot [45]. In in-direct photolysis methods of hydrogen production is predicted to be 1220$ per GJ/year. By indirect biophotolysis of hydrogen production, the capital cost is predicted to be 2.4$/gigajoule/year [91].

5. Trends in biohydrogen production and conclusions

Biohydrogen production process generally faces two bottlenecks: (1) Low hydrogen yield in dark (2) high energy cost in case of photo-fermentation. The dark fermentation process yields only 4 mol of hydrogen per mole of glucose, whereas photo-fermentation produces 12 mol of hydrogen per mole of glucose. However, photo-fermentation requires external source of light energy. The researchers have proposed two-stage processes by integration dark fermentation with photo-fermentation (Fig. 4). In dark-photo fermentation model 4 mol of hydrogen can be produced under dark and rest of the byproducts can be oxidized by photosynthetic bacteria to produce hydrogen. Another approach to degrade acetate (an intermediate product) is to use acetate containing biomass in microbial fuel cell (MFC) to produce 8 mol of hydrogen. Produced proton at cathode by fermentative bacteria will be reduced at cathode to produce hydrogen [92]. Logan et al. developed a method of electricity generation using fuel cell microbial, via acetate containing biomass [93]. This novel MFC referred as bio-chemically assisted microbial reactor (BEAMR) has potential to generate pure hydrogen gas at the cathode. Domestic wastewater could be used as substrate. This way efficient and sustainable hydrogen production using microalgae is possible [93]. Another approach is to produce methane from these byproducts than hydrogen but the output efficiency is not explored yet.

Wastewater treatment by the use of microalgae has been investigated since long; however, the application is not commercialized yet. A wide variety of microalgal species are able to grow in wastewaters. The main difficulty to grow microalgae in wastewater is the presence of high concentration of ammonia which inhibits microalgae growth. Furthermore, it is required to determine whether or not this process is truly sustainable and carbon neutral in terms of the utilization [94–96].

Metabolic engineering is a tool to bring a major breakthrough in biohydrogen process. By exploring the pathway of hydrogen production using molecular biology, this technique can eliminate bottlenecks, and increase carbon flow to hydrogen-producing pathway. It can also be helpful to increase the substrate utilization by engineering more efficient and oxygen resistant hydrogen

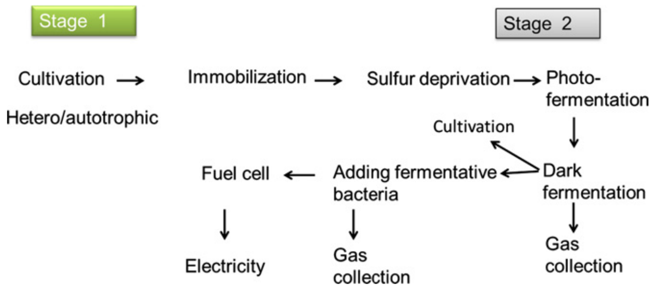


Fig. 4. A concept for enhanced hydrogen production and electricity generation by microalgae.

evolving enzymes [19]. The *C. reinhardtii* genome sequence showed several unexpected pathways, such as inorganic carbon fixation, fermentation, and vitamin biosynthesis [19,61,66]. Each of them can be exploited to improve the biohydrogen yield. Exploring the effects of nutrients limitation and substrate utilization can benefit to discover particular chromosomal genes in microalgae for enhanced hydrogen production [19]. Random and direct mutagenesis has succeeded in improving tolerance by 10-fold. One approach to address this problem is gene shuffling, which has been used to generate a diverse recombinant hydrogenase library to screen for enhanced O_2 tolerance and stability [19,68]. A new strategy has been introduced to search natural diversity through the use of degenerate PCR primers [19].

Developments are required for optimum design of photobioreactors. Another critical issue is to find a cheaper carbon source that could produce hydrogen efficiently. To address the economy of this process, the shortening of the total time of hydrogen production should be on top priority. The use of optical fiber is a striking approach to decrease the lag time for hydrogen production. Biological hydrogen is currently more expensive than other fuels. Thus, if technology improvements succeed in bringing down the costs, it can attain considerable attention as a sustainable biofuel. The optimization of key experimental factors, genetic modification, and metabolic engineering of microalgae are the ultimate approaches to make hydrogen production cost-effective and sustainable.

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